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Amino-Terminal Epitopes are Exposed When Full-Length Open Reading Frame 2 of Hepatitis E Virus is Expressed in *Escherichia coli*, But Carboxy-Terminal Epitopes are Masked

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We constructed a panel of overlapping and non-overlapping fragments of cDNA derived from open reading frame 2 (ORF2) of hepatitis E virus (HEV) and fused to the gene encoding glutathione *S*-transferase (GST), from which proteins were expressed in *Eschericia coli.* IgG-specific immunoreactivity against each protein was measured by Western immunoblotting using sera from experimentally infected Rhesus macaques (*Macaca mulatta*) or from HEV-infected patients. Under these conditions, full-length ORF2 protein (GST-ORF2) was strongly reactive with acute-phase sera from either macaques or patients, but was poorly reactive with convalescent sera. Recombinant protein GST-ORF2.3, representing amino acids 1–110 of the 660 encoded by ORF2, demonstrated a pattern of reactivity largely indistinguishable from the full-length protein. Conversely, GST-ORF2.1, representing amino acids 394–660 of the ORF2 protein was strongly reactive with both acute- and convalescent-phase sera. Extension of GST-ORF2.1 towards the N-terminus led to a progressive loss of convalescent-phase reactivity, apparent with as few as 20 additional HEV-specific amino acids. Deletion of 40 or more amino acids from the N-terminus of ORF2.1 also led to reduced convalescent-phase reactivity, however a protein representing this ''reactive'' region, containing amino acids 394–473, was poorly reactive, suggesting that the convalescent-reactive epitopes are conformational. Expression of full-length ORF2 protein in *E. coli* therefore masks the convalescent-reactive epitopes within the C-terminal part of the protein, without affecting N-terminal, acute-reactive epitopes. ***J. Med. Virol. 52:289–300, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: HEV; antigen folding; convalescent reactivity; acute reactivity

INTRODUCTION

Hepatitis E virus (HEV) is responsible for epidemic and sporadic hepatitis E (HE), and is a major public health problem in developing countries. More recently, sporadic cases of hepatitis E have also been reported in some developed countries [Bradley, 1992; Dawson et al., 1992; Lok et al., 1992; Coursaget et al., 1993; Coursaget et al., 1994; Tassopoulos et al., 1994; Zanetti et al., 1994; Heath et al., 1995]. HEV is a single-stranded, positive sense, polyadenylated RNA virus. It has tentatively been classified as a member of the *Caliciviridae* [Bradley, 1992; Krawczynski, 1993; Cubitt et al., 1995], although it has also been noted to share a number of features of genome organisation in common with the superfamily of Alphaviruses [Koonin et al., 1992; Purdy et al., 1993]. The genome contains three open reading frames (ORFs) with ORF2 encoding the putative major structural or capsid protein. The cloning of geographically distinct strains of HEV and synthesis of recombinant HEV proteins [Reyes et al., 1990; Ichikawa et al., 1991; Tam et al., 1991; Yarbough et al., 1991; Aye et al., 1992; Huang et al., 1992; Uchida et al., 1992; He et al., 1993; Li et al., 1994] along with the development of animal models for HE infection [Gupta et al., 1990; Bradley, 1992; Jameel et al., 1992; Tsarev

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et al., 1992, 1993, 1995; Arankalle et al., 1993; Longer et al., 1993] have made it possible to study antigenic and immunogenic epitopes of HEV proteins. In addition, the expression of ORF2 and ORF3 proteins in *E. coli* and Baculovirus systems, and the production of synthetic peptides have led to the development of diagnostic assays [Dawson et al., 1992; He et al., 1993; Tsarev et al., 1993; Yarbough et al., 1993; Favorov et al., 1994; Khudyakov et al., 1994; Paul et al., 1994]. However much more work is needed in the development of diagnostic assays appropriate for the countries in which HEV infection is endemic, and the currently available assays vary widely in their sensitivity and specificity [Mast et al., 1996].

We reported previously a Western blot assay using glutathione *S*-transferase (GST)-HEV fusion proteins representing full-length ORF3 (GST-ORF3) and the carboxy-terminal 267 amino acids of ORF2 (GST-ORF2.1) to detect IgG anti-HEV [Li et al., 1994]. Using this assay, antibody to GST-ORF3 was detected in some experimentally infected monkeys within 17 days of inoculation, but this reactivity waned rapidly beyond 60 days to undetectable levels. Antibody to GST-ORF2.1 was also detected early but, unlike antibody to GST-ORF3, was present in every animal and persisted at high levels for over 12 months, during which time animals were rechallenged with virus without developing obvious signs of hepatitis. This suggests that the carboxy-terminal region of HEV ORF2 contains epitopes which may be associated with humoral immunity to reinfection.

Purdy et al. [1993] first reported that immunisation of two cynomolgus macaques with a *trp*E-HEV ORF2 fusion protein, representing the carboxy-terminal two-thirds of the putative capsid protein, prevented development of hepatitis in these primates after challenge by wild-type HEV. More recently Tsarev et al. [1994a,b] reported protection of cynomolgus monkeys against hepatitis after challenge with virulent HEV, by both passive and active immunisation of these monkeys with ORF2-specific antisera or protein, respectively. However, the relationship between antibody reactivity, the different recombinant antigens used, and immunity to reinfection is still poorly understood. A greater understanding of these relationships would clearly advance efforts towards the control of HEV infection.

Questions remain regarding the use of *E. coli*-expressed proteins and synthetic peptides derived from HEV due to wide variability in the detection of antibody in reference sera [Mast et al., 1996] or epidemic sera [Clayson et al., 1995], but the possible reasons for discrepancies between such assays using recombinant ORF2 proteins have not previously been addressed. A surprising finding of our earlier study was the observation of reduced convalescent reactivity in a larger fragment of ORF2 which contained the entire sequence of the 2.1 fragment [Li et al., 1994], and we reasoned that such an effect might contribute to the problems encountered with HEV antigens expressed in *E. coli*. To define better the antigenic epitopes within ORF2 and to resolve the effect of extended amino acid sequences on this reactivity, we constructed a series of clones encoding fusion proteins representing overlapping and non-overlapping fragments of ORF2, in pGEX (GST) and PinPoint expression systems. The fusion proteins were used in Western blot assays to detect antibody reactivity in serial sera from monkeys experimentally infected with HEV and in sera from humans with acute or past hepatitis E.

MATERIALS AND METHODS

Construction of ORF2 Plasmids for Expression

The clones used in this study are shown in Figure 1. Three clones, pGEX1-AC3, -AC2.1, and -AC2.2, were reported previously [Li et al., 1994]. To construct full-length ORF2, we utilised the overlapping portions of clones AC3 and AC2.2. The 5′-end 331bp of ORF2 (AC2.3) was amplified from AC3 using a primer pair (P4740 and P3566) containing *Bam* HI or *Eco* RI restriction sites to facilitate cloning into pGEX1 (Pharmacia Biotech, Uppsala, Sweden). The second primer pair (P2 and P5019) was designed to amplify a 440bp fragment of ORF2 from AC2.2 which overlapped AC2.3 by 34 nucleotides. DNAs from pGEX1-AC3 and pGEX1-AC2.2 were extracted by standard plasmid DNA mini-preparation and used as templates for PCR to amplify the two overlapping fragments. The AC2.3 PCR product, containing the 331bp at the 5′-end of ORF2, was digested with *Bam* HI and *Eco* RI and ligated with pGEX1, to generate the clone pGEX1-AC2.3. The second PCR product, from pGEX1-AC2.2, was digested with *Pvu* II and *Eco* RI and the 360 bp product was ligated with a restriction fragment of 312bp generated from ORF2.3 after *Bam* HI and *Pvu* II digestion. This fragment was then ligated into pGEX1 to produce the clone pGEX1-AC2.4 which contained 672 bp at the 5′ end of ORF2. pGEX1-AC2.4 was then digested with *Eco* RI and ligated with a 1328bp *Eco* RI fragment from pGEX2.2 to give pGEX-AC2, which contains full length ORF2 protein in frame with GST.

The serial deletion- and addition clones were constructed by PCR using pGEX-AC2 as a template, to remove certain portions from the 5′ end of ORF2. Another clone, pGEX3-ACC2, was generated by digesting a full-length ORF2 clone with *Eco* RI to recover a 1320bp fragment which was then cloned into the pGEX3 vector. Among these clones, 406.3-2+, SG3+, and C2 are based on ORF2 fragments previously described by others [Yarbough et al., 1991, 1993; Purdy et al., 1992]. However, as our constructs terminate at the stop codon in ORF2 both 406.3-2+ and SG3+ contain the five C-terminal amino acids which are not present in the 406.3-2 and SG3 proteins.

Table I displays the designation of the clones in pGEX system used in this study, the positions in ORF2 encoded by these clones, and the sequence of primers used in PCR amplifications.

To generate clones in PinPoint Xa3 vector (Promega, Madison, WI), which yield biotinylated fusion proteins

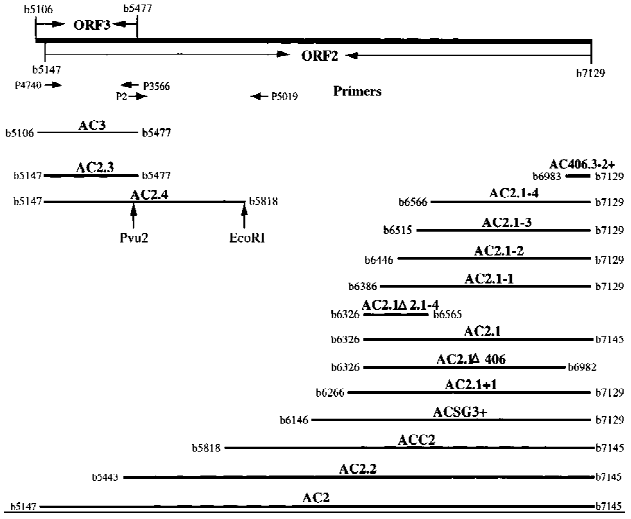

Fig. 1. HEV-specific cDNA inserts in the clones used in this study. Arrows show the positions of primers and restriction sites used in cloning strategy.

in *E. coli,* pGEX-AC2.4 and pGEX-AC2 were digested with *Bam* HI and *Eco* RI then cloned into pCR™ II vector (Invitrogen, Leek, The Netherlands), for subsequent cloning strategies. AC2.4 and AC2 fragments were released from pCR(R) II plasmids by digesting with *Bam* HI and *Eco* RV, and ligated with PinPoint Xa3 vector. The resulting PinPoint Xa3 plasmids containing AC2.4 and AC2 were designated as PP3-AC2.4 and PP3-AC2, respectively. PP-AC2 was digested by *Pvu* II to remove the 5′-end 1179 nucleotides of ORF2, and religated to give the plasmid PP-AC2.1.

The PCR and construction of above clones were carried out according to the methods described previously [Li et al., 1994]. All clones were screened by restriction enzyme digestions for the presence of inserts of the expected sizes. Selected colonies from each transformant were then induced with isopropylthio-β-D-galactoside (IPTG) and examined for expression of the fusion proteins of the expected sizes.

Production of GST- and Biotinylated Fusion Proteins

The transformants of clones in pGEX vectors were cultured in LB medium containing 50 μg of ampicillin per ml for 3–5 hr. IPTG was added to a final concentration of 1 mM, and the culture was grown for another 5 hr. The transformants of PP-AC2.1, PP-AC2.4, and PP-AC2 were cultured in LB medium containing 2 μM biotin and 100 μg of ampicillin per ml for 1 hr then induced with 0.1 mM IPTG as above. The fusion proteins formed inclusion bodies and were purified as follows. Cells were pelleted at 3,500 rpm for 5 min in a GS-6 Centrifuge (Beckman Instruments, Fullerton, CA). For each gram of packed cells, 1 ml of lysis buffer (50 mM Tris pH 8.0, 1 mM EDTA, 100 mM NaCl) was added and the pellet was gently resuspended. Lysozyme (Boehringer-Mannheim GmbH, Mannheim Germany) was added to a final concentration of 1 mg/ml and the suspension was incubated at 4°C for 30 min. Sodium deoxycholate was added to a final concentration of 0.1%. The suspension was stirred continuously at room temperature for approximately 5 min. The solution was then incubated with 20 U/ml of DNase (Promega) at 37°C until it was no longer viscous (approximately 20 min). The inclusion bodies were pelleted by centrifuging at 13,000 rpm (Beckman) for 15 min and then washed with lysis buffer containing 0.5% Triton X-100. The pellet was resuspended in a volume equivalent to 1/500th of the initial culture volume and stored in aliquots at –20°C.

SDS-PAGE and Western Blot

In brief, each of the fusion proteins was resuspended in gel loading buffer (50 mM Tris pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 0.01% bromophenol blue, 8% glycerol), boiled for 2 min and subjected to 10% SDS-PAGE. The proteins were transferred to nitrocellulose membranes after electrophoresis. The membranes were blocked with 3% casein in TBST (0.1 M Tris pH 7.5, 0.1 M NaCl, 0.3% Tween-20) and then probed with serial sera from monkeys or human sera, diluted 1:500 in TBST containing 1% casein. The membranes were counterstained with HRPO-conjugated rabbit anti-human IgG (Dako Co., Glostrup, Denmark) and enzyme complexes were detected by enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, UK) [Li et al., 1994].

Test Sera for Western Blot

(i) Macaque sera. Seven rhesus monkeys (M1, M2, M27, M31, M32, M33, M34) were inoculated intravenously with the Chinese strain of HEV [Aye et al., 1992]. Among them, M31 and M32 were rechallenged at days 53, 107, and 316, and M33 was rechallenged at days 107 and 316 after initial infection. Serum specimens were collected weekly up to 360 days (M31, M32, M33) or 429 days (M1) postinfection and stored at –20°C. The serum samples from other monkeys (M2, M27, and M34) up to 22, 90, and 48 days postinfection, respectively, were available for analysis. Bile specimens were collected from two monkeys (M1, M2) at the estimated peak of ALT levels.

(ii) Human sera. Twenty-three acute-phase and twelve convalescent-phase sera were collected from patients within 3 months or between 3 and 9 months after onset of the disease, respectively, during a hepatitis E epidemic in Xinjiang, China. This is a subset of 66 acute and 19 convalescent sera reported previously [Li et al., 1994], with insufficient sample remaining in the other sera. Ten serum specimens were from non-A, non-B, non-C acute hepatitis patients admitted to Fairfield Hospital, Australia, all of whom had a history of travel to developing countries prior to the onset of illness. Two convalescent hepatitis E sera were part of a panel of well-characterised sera provided by Centers for Disease Control and Prevention/National Institutes of Health, USA [Mast et al., 1996]. Negative control sera included samples from ten patients with acute

TABLE I. Designation of Clones, Amino Acid Positions of Proteins, and Primer Sequences in pGEX Expression System

Clones	Amino acid position in ORF2	No. of amino acid	Fusion proteins (KDa)	Primers[a]	
pGEX1-AC2.3	1-110	110	GST-ORF2.3 (38)	Forward (4740):	5' - G T G G A T C C A T G C G C C C T C G G C C T A T T T T G T -3'
				Reverse (P3566):	5' - G T G A A T T C T T A G C G G C G C G G C C C C A G C T -3'
pGEX1-AC2.4	1-224	224	GST-ORF2.4 (50)	[b]	
pGEX1-AC406.3-2+	613-660	48	GST-406.3-2+ (32)	Forward:	5'-T T G C T T G G A T C C A C C A T G G A C T A C C C T G C C -3'
pGEX1-AC2.1-4	474-660	187	GST-ORF2.1-4 (46)		5'-G T G C T T G G A T C C T C T C T C A C C G C T G C C G A G T A T G -3'
pGEX1-AC2.1-3	457-660	204	GST-ORF2.1-3 (48)		5'-A C A C C T G G A T C C G C C C C A T C G C G C C C T T T T T C -3'
pGEX1-AC2.1-2	434-660	227	GST-ORF2.1-2 (51)		5'-G A C A T C G G A T C C G G G G G A G T C T C G T G T A G T T A T T C -3'
pGEX1-AC2.1-1	414-660	247	GST-ORF2.1-1 (53)		5'-G T T A A G G G A T C C A C A T C T G T A G A G A A T G C T C A G C A G G -3'
pGEX1-AC2.1	394-660	267	GST-ORF2.1 (56)		5'-G T G A A T T C C A G C T G T T C T A C T C C C G T C C -3'
pGEX1-AC2.1 + 1	374-660	287	GST-ORF2.1 + 1 (57)		5'-C T T A C C G G A T C C A A C C T T G C T G A C A C C C T G C T T G -3'
pGEX1-AC2.1 + 2	356-660	305	GST-ORF2.1 + 2 (59)		5'-G A C C T C G G A T C C A C T A G T A C T A A T G G T G T C -3'
pGEX1-ACSG3+	334-660	327	GST-SG3+ (62)		5'-C G C C T T G G A T C C G G T G C A G A T G G G A C T G C C G A G C T T A -3'
				Reverse:	5'-A A G C A A G G A T C C C T A T A A C T C C C G A G T T T T A C C C A C C -3'
pGEX3-ACC2	225-660	436	GST-C2 (74)		
PGEX1-AC2	1-660	660	GST-ORF2 (98)		
pGEX1-AC2.1Δ406	394-612	219	GST-ORF2.1Δ406 (50)	Reverse:	5'-G T A G T C G A A T T C A T C C T C A A G C A A T G C T A G C G C G -3'
pGEX1-AC2.1Δ2.1-4	394-473	80	GST-ORF2.1Δ2.1-4 (35)		5'-A G C G G T G A A T T C A A G C C A A A G C A C A T C A T T A G C -3'
				Forward:	5'-G T G A A T T C C A G C T G T T C T A C T C C C G T C C -3'

[a]The primers were modified to contain EcoR1 or BamH1 restriction sites to facilitate cloning. The HEV-specific sequences are underlined.
[b]Primers, to amplify a 440bp fragment of ORF2 from AC2.2 then generate pGEX1-AC2.4, are: Forward (P2): 5'-TCGTAGACCTACCACAGCTG-3'; Reverse (P5019): 5'-CACAAGCTCAGAGGCTATGCCG-3'.

hepatitis A, ten with acute hepatitis B, ten with hepatitis C, five with Epstein-Barr virus (EBV), and five with cytomegalovirus (CMV) infection.

Densitometry of Western Blot Results

The reactivity of sera against each of the recombinant proteins in Western blots was measured by densitometry (Nikon Scan Macintosh) and analysed using the NIH Image programme.

RESULTS

Expression of HEV Fusion Proteins

GST fusion proteins were produced by induction with IPTG from the transformants of constructs in pGEX system. The estimated molecular weights of the protein products were consistent with the predicted sizes of proteins encoded by these clones, including the 26 kDa of GST (Table I). The corresponding biotinylated fusion proteins produced in the PinPoint Xa3 system were PP-ORF2.1, PP-ORF2.4, and PP-ORF2, with the approximated molecular weights of 44.5, 42, and 85.5 kDa, respectively, including 13 kDa for the biotinylation tag.

Detection of Anti-HEV in Macaque Sera Using GST Fusion Proteins

All seven rhesus monkeys had an elevated level of ALT around 10 to 30 days after the initial infection, consistent with acute hepatitis. RT-PCR for bile specimens from two monkeys showed the presence of HEV RNA (results not shown). The three monkeys which were rechallenged (M31, M32, and M33) [Li et al., 1994], showed moderately elevated levels of ALT after the first rechallenge (results not shown), but we cannot exclude the possibility that this represented natural variation in the animals. Second and third rechallenges of these animals had less effect on ALT level, suggesting that the animals acquired at least partial protective immunity after one or at most two exposures to virus.

To examine the antibody reactivity of fusion proteins representing different parts of ORF2, proteins were separated by SDS-PAGE and used to detect anti-HEV IgG in Western blots using serial sera from the above monkeys.

(i) Antibody reactivity against GST-ORF2.3 and GST-ORF2.4. Two fusion proteins representing amino-terminal sequences of ORF2 were used in Western blot assays with serum specimens from the above monkeys. Both anti-GST-ORF2.3 and anti-GST-ORF2.4 were first detected 9–24 days after infection in all seven monkeys (M1, M31, M32, M33 in Fig. 2; M2, M27, M34 data not shown). The antibody reactivity peaked at 20–60 days in six of seven monkeys, while sera from M27 maintained a high level of both antibodies until day 90. Anti-GST-ORF2.3 and anti-GST-ORF2.4 then rapidly declined (M1, M31, M33) or remained at the same level (M32), indicating a strong acute phase reactivity. The samples of later bleeds from M27 and M34 also showed a decline in the level of both antibodies. Later serum specimens were not available from M2.

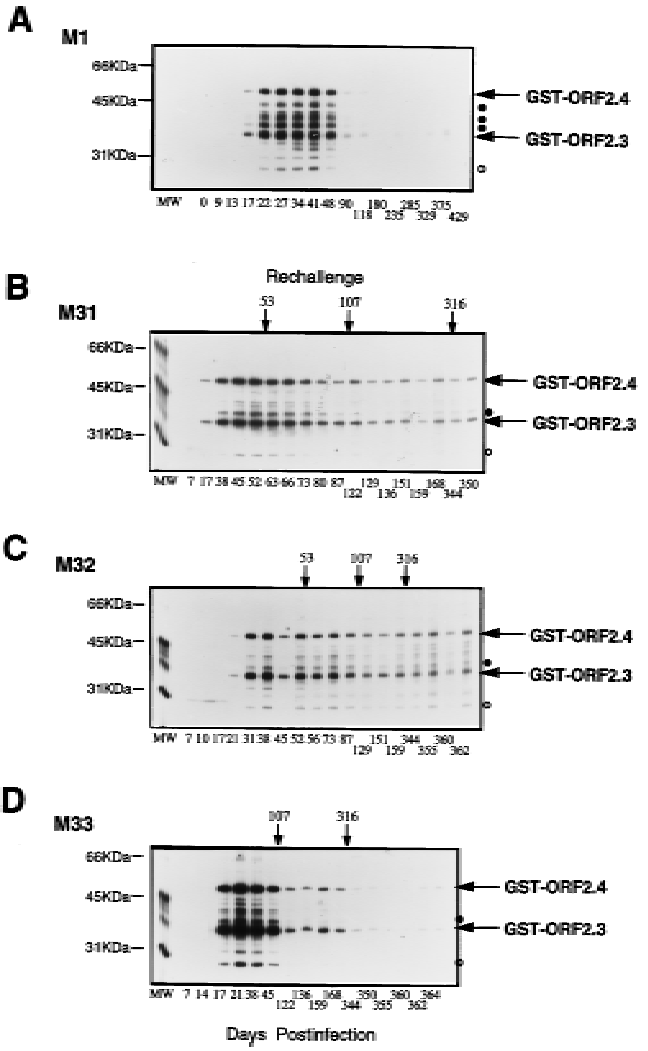

Fig. 2. Western blot of GST-ORF2.3 and GST-ORF2.4 with sera from infected macaques M1 (**A**), M31 (**B**), M32 (**C**), and M33 (**D**). Approximately equal masses of each recombinant protein and unfused GST were mixed and electrophoresed, the proteins were transferred to nitrocellulose, and antibody reactivity detected by enhanced chemiluminescence. The days of rechallenge and the days of serum collection are indicated (above and below the lanes, respectively). Lanes MW: Biotinylated molecular mass markers (sizes indicated on the left). The positions of GST-ORF2.3 and GST-ORF2.4 and their reactive degradation products (open and closed circles, respectively) are indicated on the right.

(ii) Antibody reactivity against GST-ORF2.1 and GST-ORF2. We reported previously that antibody reactivity to GST-ORF2.1 (representing the carboxy-terminal 40% of ORF2) persists at a high level until the last available specimens in all infected rhesus monkeys [Li et al., 1994]. The comparison of anti-GST-ORF2.1 and -ORF2 in M1, M31, M32, and M33 is shown in Figure 3. Antibody to the full-length GST-ORF2 was first detected in all four monkeys at the time of first detection of anti-ORF2.1 but remained at the same level (M32) or significantly declined with time (M1, M31, M33). In contrast, the level of antibody to

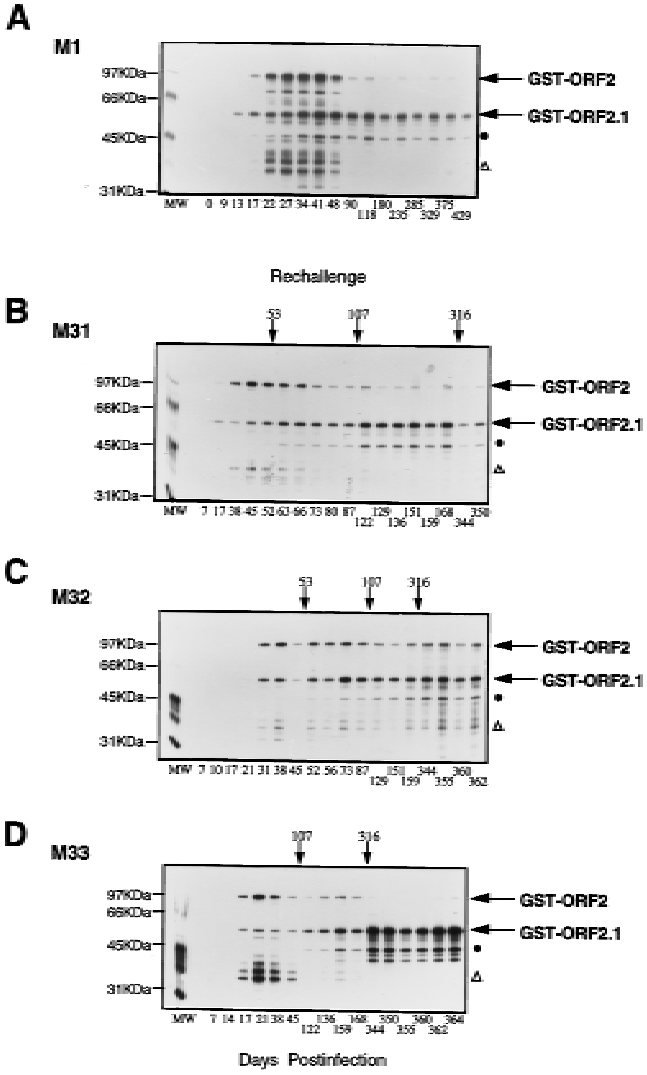

Fig. 3. Western blot of GST-ORF2.1 and GST-ORF2 with sera from infected macaques M1 (**A**), M31 (**B**), M32 (**C**), and M33 (**D**). The days of rechallenge and the days of serum collection are indicated (above and below the lanes, respectively). Lanes MW: Biotinylated molecular mass markers (sizes indicated on the left). The positions of GST-ORF2.1 and GST-ORF2 and their reactive degradation products (closed circle and open triangle, respectively) are indicated on the right.

GST-ORF2.1 continued to increase throughout the study period in those animals which were rechallenged with HEV (M31, M32, and M33) and was maintained at a steady, high level in M1 which was not rechallenged. Some of the acute phase sera (for example M1 days 22–48, M33 days 17–45) were found to be strongly reactive with small degradation products of GST-ORF2; these reactive proteins were barely visible by staining the nitrocellulose membrane with amido black and most likely represented amino-terminal sequences of ORF2 protein (see above).

These data show that full-length GST-ORF2 was strongly reactive with acute-phase but not convalescent sera from the monkeys. Comparison of the reactivity of each serum from M1, M31, M32, and M33 against the amino-terminal proteins GST-ORF2.3 and ORF2.4 (Fig. 2) and full-length GST-ORF2 (Fig. 3) shows remarkable similarities in both the acute and convalescent phase, which suggests that the amino terminus of ORF2 is largely responsible for the reactivity observed with full length ORF2. The reactivity to the whole GST-ORF2 thus mainly reflects the response to amino-terminal but not carboxy-terminal sequences of ORF2 protein.

(iii) Optimisation of the ORF2 fragment for convalescent immunoreactivity. To map precisely the region in the carboxy-terminal part of HEV ORF2 protein which was able to react strongly with convalescent antisera, fusion proteins produced from serial deletion- and addition clones (relative to GST-ORF2.1) were utilised to detect anti-HEV in serial sera from two experimentally infected monkeys (M31, M32). Figure 4 shows the immunoblots of each protein with the serial sera, demonstrating the pattern of immunoreactivity for each protein and the overall level of reactivity. However, while these immunoblots represent equivalent film exposures, we are cautious in comparing the intensities of individual bands between separate immunoblots. Accordingly, the level of immunoreactivity for each protein was estimated by densitometry of bands at day 80 (representing late acute phase) and days 159 and 360 postinfection (representing convalescent phase), with each expressed as a percentage of the reactivity observed at 63 days postinfection. In this way, the relative reactivity for convalescent- vs. acute-phase sera can easily be observed (Fig. 5). As shown in Figure 4A, two extreme carboxy-terminal proteins, GST-ORF406.3-2+ and -ORF2.1-4, reacted strongly with acute phase sera (days 38–80) after which the reactivity waned quickly to undetectable levels in M31, and only reacted with serum at day 45 in M33. The detection of epitope(s) at the extreme carboxy-terminus of ORF2, associated with acute phase reactivity, is consistent with previous reports and the use of the 406.3-2 antigen in widely used, commercial anti-HEV assays [Yarbough et al., 1991; Dawson et al., 1992].

Immunoreactivity with convalescent antisera became pronounced only when the protein extended at least 227 amino acids from the carboxyl terminus (2.1-2) and was maximal in 2.1 (267 amino acids) (Figs. 4, 5). Antibodies to GST-ORF2.1-3 and -ORF2.1-2 peaked at days 38–159 before declining in M31. In M32, antibodies to both proteins were positive for samples up to day 360. While reactivity with convalescent serum increased when longer proteins (GST-ORF2.1-1 and -ORF2.1) were expressed, it is noted that the reactivity of these proteins with very early acute-phase sera was slightly lower than that of both smaller and larger constructs. GST-ORF2.1+1, with an extra 20 amino acids added to ORF2.1, presented a slight increase of acute reactivity but a substantial decrease of convalescent reactivity. Similarly, GST-ORFSG3+ and -C2, encoding 327 and 436 amino acids, respectively, demonstrated acute-phase reactivity equivalent to that of 2.1, but with greatly reduced convalescent-phase reactivity.

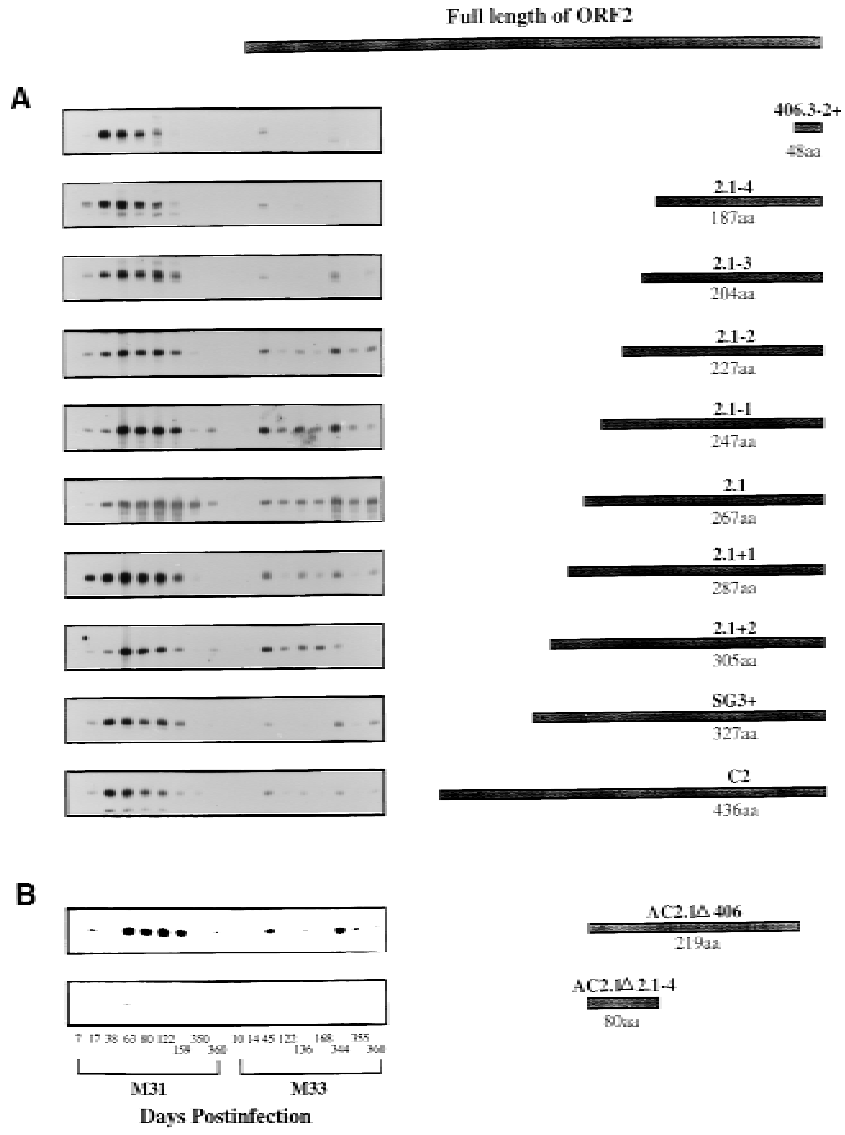

Fig. 4. Western blot of fusion proteins produced by 5′ end deletion- or addition (**A**) and 3′ end deletion (**B**) relative to the ORF2.1 fragment, using sera from infected macaques M31 and M33. The days of serum collection are indicated below the blots. The positions of each protein in ORF2 are indicated on the right.

The region between amino acids 394 and 457 (2.1 and 2.1-3) therefore contributes to the formation of epitope(s) reactive with convalescent antibody, while extension towards the N-terminus masks the formation of these epitopes.

To determine whether the "convalescent-reactive" epitopes could be formed in the context of smaller fragments of ORF2, two further clones were constructed (pGEX1-AC2.1Δ406 and -AC2.1Δ2.1-4) and corresponding proteins expressed. GST-ORF2.1Δ406, containing amino acid sequences of GST-ORF2.1 with the deletion of the 48 amino acids of the 406.3-2+ antigen, gave a pattern of reactivity in both monkeys similar to that of GST-ORF2.1. Surprisingly, protein GST-ORF2.1Δ2.1-4, spanning the region mapped to be essential for convalescent reactivity, displayed poor reactivity to both acute and convalescent sera (Fig. 4B). Although these experiments cannot determine whether the additional sequences toward the carboxyl terminus contribute to the structure of the epitope or rather to correct folding of sequences which are present in the 2.1Δ2.1-4 protein, it is clear that reactivity with convalescent-phase sera is due to conformational rather than linear epitope(s) in GST-ORF2.1, and the 48 amino acids at the carboxyl terminus do not contribute markedly to this reactivity.

Detection of Anti-HEV IgG in Human Sera

Sera from patients with acute hepatitis A, B, C, E, and EBV or CMV infection were tested for antibodies to GST-ORF2.1, GST-ORF2.3, GST-ORF2.4 and GST-ORF2 by Western blots (Table II). None of the 10 hepatitis B, 5 EBV and 5 CMV sera were positive for any

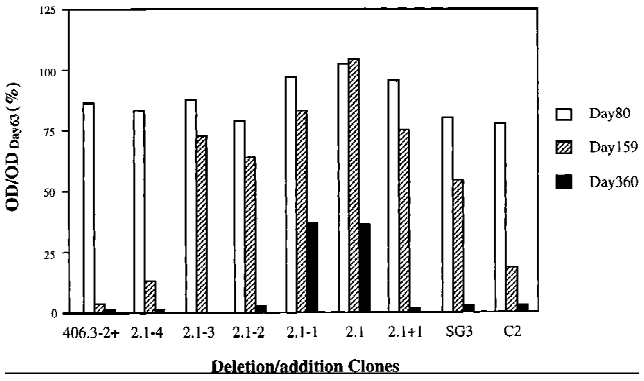

Fig. 5. Reactivity of ORF2 fragments relative to acute-phase reactivity. The signal for each protein with sera from M31 at days 80 (□), 159 (▨), and 360 (■) was quantitated by densitometry and is expressed as a percentage of the reactivity of each protein with sera at day 63.

antibodies to the four fusion proteins, while 1 of 10 hepatitis A and 1 of 10 hepatitis C sera were weakly reactive to GST-ORF2.1 and GST-ORF2.4, respectively. The positive rates of anti-GST-ORF2.1 among HE patients less than 3 months and 3 to 9 months after the onset of clinical hepatitis during a hepatitis E epidemic in China were 91% and 50% respectively, while both anti-GST-ORF2.3 and -ORF2.4 were 78% in the (less than 3 months) sera and 17% in the (3 to 9 months) sera. The rates of antibody to GST-ORF-2 (83% and 25%, respectively) were slightly higher than anti-GST-ORF2.3 and -ORF2.4. Among 21 acute-phase sera positive for one or more fragments of ORF2, 17 were positive for all four proteins, two for anti-GST-ORF2.1 and -ORF2, one for anti-GST-ORF2.1, and one for anti-GST-ORF-2.1, -ORF2.3, and -ORF2.4. Of 10 acute non-A, non-B, non-C hepatitis cases, who had travelled to developing countries and had an onset of acute hepatitis within 6 weeks prior to collection of sera, nine were positive for anti-GST-ORF2.1 and -ORF2.3, and eight of these were also positive for anti-GST-ORF2.4 and -ORF2. These results indicate that the two amino-terminal fusion proteins, GST-ORF2.3 and -ORF2.4, are strongly reactive with only acute phase sera and may be detectable for only a short time. With acute-phase sera, full-length GST-ORF2 has a rate of reactivity similar to ORF2.3 and ORF2.4, although the level of reactivity for each was roughly equivalent in patients' specimens (data not shown), in contrast to the higher reactivity of the amino terminal parts of ORF2 observed with macaque sera (Fig. 2).

To compare further the levels of antibody reactivity with GST-ORF2.1 and GST-ORF2, sera from one acute- and two convalescent-phase patients were serially diluted with negative control sera and reacted with each protein in immunoblots (Fig. 6). GST-ORF2.1 and GST-ORF2 were able to detect anti-HEV in the acute-phase serum at dilutions of 1:8000 and 1:4000, respectively, suggesting the presence of similar levels of antibody to each protein in acute-phase serum. In contrast, while the two convalescent sera (from patients 1.5–2 years and 13 years after infection) reacted with GST-ORF2.1 at dilutions of 1:4000 and 1:8000, respectively, against GST-ORF2 one serum was negative at all dilutions and the other only weakly positive at a 1:2000 dilution. Antibody to the ORF2.1 fragment clearly persists at higher levels than that to the full-length ORF2 protein under the conditions tested.

Antibody Reactivity Against PinPoint Fusion Proteins

To determine whether the fusion protein component, GST, contributed to the masking of convalescent-phase reactivity in proteins containing viral sequences amino-terminal to the 2.1 fragment, we constructed the corresponding clones encoding the amino-terminal (2.4), carboxy-terminal (2.1), and full-length ORF2 fragments in the PinPoint expression system which contains a tag of only 128 amino acids (compared to 220 amino acids in GST). Immunoblots of the three biotinylated fusion proteins, PP-ORF2.1, PP-ORF2.4 and PP-ORF2, were used to detect anti-HEV IgG in serial sera from M31 and M33, with the reactivities being essentially identical to that of the corresponding GST fusions (data not shown). Similar results have been seen in preliminary studies in the pET expression system with fusions of only 50–75 amino acids (data not shown). The masking of convalescent-phase reactivity therefore appears to be independent of the fusion protein used for expression in *E. coli;* rather, it results from the inclusion of virus-specific amino acids additional to those represented in the ORF2.1 constructs.

DISCUSSION

The routine diagnosis and seroepidemiological studies of HEV infection remain largely dependent on the detection of antibody using recombinant viral proteins. However, the antibody response to HEV-derived antigens varies markedly between individual antigens and between individual patients, thus complicating such studies [Mast et al., 1996]. The putative viral capsid protein, encoded by ORF2, appears to elicit variable responses in both patients and experimental animals, but the extent to which this reflects differences in virus strains, the antigens used in assays, or rather in individual antibody responses is not well understood. We have now shown that within a single virus strain and infected individual, variable responses to the ORF2-derived protein can be observed as a result of perturbations in protein folding elicited by viral sequences present in the full-length protein, at least in a Western blot. It is therefore clear that the choice of ORF2 fragments for expression is critical for an improved understanding of HEV immunity.

Immunoblotting with serial sera from experimentally infected animals revealed distinct conformational epitopes in the carboxy-terminal 40% and the amino-terminal 17% of the molecule, represented by GST-ORF2.1 and GST-ORF2.3 respectively. We reported previously that the reactivity of convalescent-phase sera to GST-ORF2.1 is reduced when the protein is

TABLE II. Detection of Anti-HEV IgG by Western Blotting

Infection with	n	No. (%) positive to indicated fusion protein				
		GST-ORF2.1	–ORF2.3	–ORF2.4	–ORF2	–ORF3
Hepatitis A	10	1 (10)	0	0	0	ND[a]
Hepatitis B	10	0	0	0	0	ND
Hepatitis C	10	0	0	1 (10)	0	ND
Cytomegalovirus	5	0	0	0	0	ND
Epstein-Barr virus	5	0	0	0	0	ND
Hepatitis E						
Acute[b]	23	21 (91)	18 (78)	18 (78)	19 (83)	20 (87)
Convalescent[c]	12	6 (50)	2 (17)	2 (17)	3 (25)	3 (25)
Imported[d]	10	9 (90)	9 (90)	8 (80)	8 (80)	ND

[a]Not determined.
[b]Less than 3 months after onset of disease, from an epidemic of HE in Xinjiang, China.
[c]3 to 9 months after onset of disease, from an epidemic of HE in Xinjiang, China.
[d]Presumed acute hepatitis E: history of recent travel to developing countries, less than 6 weeks after onset of hepatitis, excluding hepatitis A, B, and C, from Fairfield Hospital, Australia.

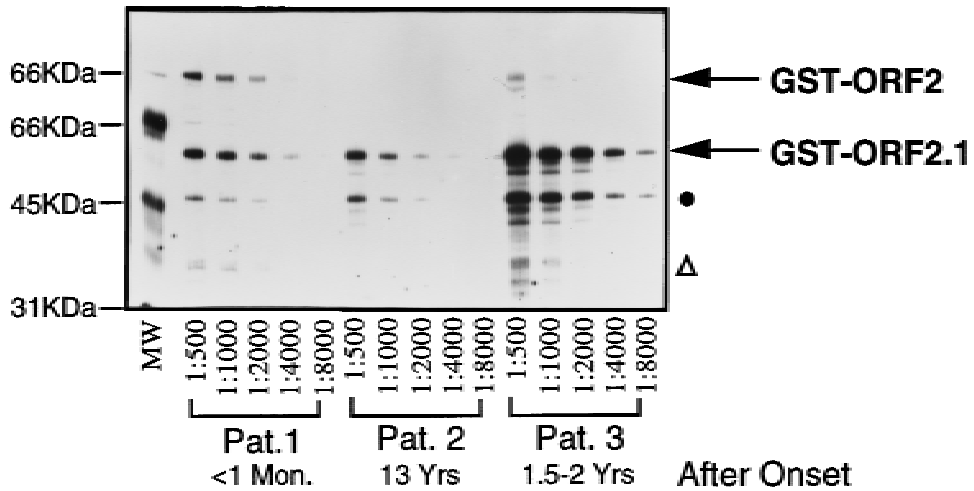

Fig. 6. Western blot of GST-ORF2.1 and GST-ORF2 with serially diluted acute and convalescent sera from HE patients. Dilutions of serum are indicated below the lanes. Lanes MW: Biotinylated molecular mass markers (sizes indicated on the left). Circle and triangle are as defined in the legend to Figure 3.

expressed as part of a large portion of ORF2 (GST-ORF2.2) [Li et al., 1994]. This suggested that the inclusion of additional viral sequences may hamper the correct folding of the protein and mask important antigenic epitopes. Correspondingly we found here that the fusion proteins even 20 amino acids longer than GST-ORF2.1 begin to lose their reactivity to convalescent-phase sera, and the reactivity of full length ORF2 mainly reflects the novel amino-terminal fragments of the protein described here (Figs. 2, 3). The results also suggest that the convalescent-reactive epitopes in the HEV ORF2 protein are non-linear, as removal of the C-terminal 187 amino acids (which are only reactive with acute phase sera) from GST-ORF2.1 abolishes protein reactivity with convalescent phase sera (Fig. 4B). In addition, the reactivities of both the amino-terminal and carboxy-terminal proteins are not affected by preincubation of sera with synthetic peptides representing major linear epitopes (25–38 and 515–530, respectively) [Kaur et al., 1992; Khudyakov et al., 1994a, data not shown].

The ORF2 fragments represented in GST-ORF2.1 and -ORF2.1-1 therefore appear to be the most suitable for detection of past infections, with the inclusion of additional viral sequences actually reducing this reactivity. Although we observed only moderate reactivity in the small number of convalescent patients in the present study (Table II) it should be noted that the GST-ORF2.1 antigen was reactive with all convalescent-phase sera in a previous study representing HEV infections from many geographic regions [Mast et al., 1996], and we believe that the use of a more standardized assay, such as an EIA based on this antigen, should allow the efficient detection of past HEV infection as observed in our experimental animals.

As is the case for past infections, the detection of acute HEV infections is complicated by variable antibody responses. Reverse transcription-PCR appears to be the most sensitive method currently available for the detection of acute HEV infection [Clayson et al., 1995], but is obviously limited in its usefulness on a large scale. Solid-phase EIAs have been developed to detect HEV-specific IgG and IgM using recombinant proteins or synthetic polypeptides from ORF2 and/or

ORF3 [Dawson et al., 1992; He et al., 1993; Tsarev et al., 1993; Favorov et al., 1994; Khudyakov et al., 1994; Paul et al., 1994]. Most of these use a carboxy-terminal ORF2 protein and/or a carboxy-terminal or whole ORF3 protein as antigen, or in one case a "mosaic" protein containing epitopes from both reading frames [Khudyakov et al., 1994], although linear epitopes have also been identified by the use of peptide scanning [Kaur et al., 1992; Khudyakov et al., 1993, 1994]. In addition, Western blot assays using recombinant ORF2 expressed in Baculovirus [He et al., 1993] or truncated proteins derived from the carboxyl terminus of ORF2 have been reported [Purdy et al., 1992; Li et al., 1994].

The rapid loss of IgG antibody against many viral antigens has in fact facilitated diagnosis of infection, as antibody to ORF3 protein or antigens such as the 406.3-2, SG3 and C2 proteins has a high predictive value of recent exposure to virus in patients from regions where HEV is uncommon. However, the persistence of antibody in some individuals [Dawson et al., 1992] presents a serious diagnostic problem in populations in which HEV is endemic [Panda et al., 1995]. Reliable IgM-based assays for HEV are therefore needed. Recently, Tsarev et al. [1993] have developed an EIA based on full-length ORF2 protein expressed in insect cells using the Baculovirus system. Using this assay, IgM and IgG anti-HEV patterns were determined in experimentally infected primates, and later in sera collected during a hepatitis outbreak in Pakistan [Bryan et al., 1994]. Although IgG reactivity was detectable for 20 months in patients, the level of reactivity was found to wane from around 2 weeks after hospitalisation, whereas reactivity in the infected primates appeared more stable. When compared with our studies in rhesus macaques, this pattern of reactivity appears similar to ORF2.1. However, since HEV endemicity is most common in developing subtropical countries, the optimal IgM assays should be robust and it is not known whether this will be true of the baculovirus-expressed HEV antigen. Immunoblot assays as described here also fail to fulfil this criteria, however we have found them useful for defining the most suitable *E. coli*-expressed antigens for further study.

Amino-terminal (GST-ORF2.3 and GST-ORF2.4) and extreme carboxy-terminal (GST-406.3-2+ and GST-ORF2.1-4) fusion proteins were found to be strongly reactive with acute but not convalescent sera, in a similar pattern to that noted previously with GST-ORF3 [Li et al., 1994]. Conversely, GST-ORF2.1 reacts strongly with both acute and convalescent sera (Fig. 3) [Li et al., 1994]. The slightly higher rate of IgG antibody detection with the 2.1 antigen observed here (Table II) suggests that this antigen offers a suitable substrate for the detection of early antibody responses, but the strong reactivity of the amino-terminal proteins is also notable. While the precise patterns of IgM reactivity to these proteins have not yet been determined, it is interesting to note that the ORF2.3 antigen does not overlap with either the trpE-C2 fragment [Purdy et al., 1992] or the truncated ORF2 proteins produced in Baculovirus expression systems [Tsarev et al., 1994; Yarbough et al., 1996], both of which have been used as experimental vaccines. This lack of overlap, which extends also to ORF2.1, may make the 2.3 antigen of particular interest in monitoring antibody responses in immunised individuals and experimental animals following exposure to virus challenge.

The immunoblot assays reported above appear to be highly specific, as none of the negative control sera were found to be reactive to GST-ORF2.3 or -ORF2. One of the 10 hepatitis A sera was weakly reactive with GST-ORF2.1 but not with the other three proteins. Given the identical routes of transmission, this may represent previous infection with HE since antibody to this protein persists in many patients [Li et al., 1994]. It should be noted that GST-ORF2.1 also contains the amino acid sequence (aa 515-530) which Khudyakov and coworkers [1994] have reported to be associated with cross-reactivity in a similar proportion of healthy blood donors, however we found that preincubation of sera with this peptide does not block the reactivity of GST-ORF2.1 (results not shown). One of the 10 hepatitis C sera was weakly reactive with GST-ORF2.4, the longer amino-terminal ORF2 protein. In the absence of other reactivities, especially anti-GST-ORF2.1 and -2.3, we believe that this represents a non-specific reaction against sequences in GST-ORF2.4 which are not present in GST-ORF2.3.

It is suggested therefore that the amino-terminal fragments of ORF2 protein may provide additional antigens useful for the detection of IgG or total antibody during acute hepatitis E, either alone or together with other acute-phase-reactive proteins (for example, ORF3 protein or the extreme carboxy-terminal part of ORF2). Anti-GST-ORF2.1 on the other hand is a more sensitive marker of previous exposure to HEV, and may also better reflect the response to protective epitopes. The 2.1 and 2.3 antigens should also prove useful in assays for IgM anti-HEV.

The results of this and our previous study suggest that the carboxy-terminal part of ORF2 contains epitopes which are highly reactive in convalescent-phase sera and thus likely to be associated with immunity to infection with HEV, but these epitopes may be masked when larger portions or the full length of ORF2 are expressed as recombinant proteins, at least in *E. coli.* Although the convalescent reactivity observed with ORF2.1 may be more pronounced in monkeys, our ongoing seroepidemiological studies in populations where HEV is endemic suggest that this reactivity does persist in man, but perhaps at lower levels (data not shown). Should the immunogenicity of the proteins reflect their antibody reactivity, immunisation with the ORF2.1 fragment of capsid protein may be more effective than with larger parts of ORF2, such as the C2 antigen [Purdy et al., 1993], in the development of highly effective vaccines against HE infection. Our ongoing studies will aim to determine the precise sequences within ORF2 responsible for antigenicity and immunity to HEV.

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REFERENCES

Arankalle VA, Favorov MO, Chadha MS, Phule DM, Banerjee K (1993): Rhesus monkeys infected with hepatitis E virus (HEV) from the former USSR are immune to subsequent challenge with an Indian strain of HEV. Acta Virology 37:515-518.

Aye TT, Uchida T, Ma XZ, Iida F, Shikata T, Zhuang H, Win KM (1992): Complete nucleotide sequence of a hepatitis E virus isolated from the Xinjiang epidemic (1986–1988) of China. Nucleic Acids Research 20:3512.

Bradley DW (1992): Hepatitis E: Epidemiology, aetiology and molecular biology. Reviews in Medical Virology 2:19–28.

Bryan JP, Tsarev SA, Iqbal M, Ticehurst J, Emerson S, Ahmed A, Duncan J, Rafiqui AR, Malik IA, Purcell RH, Legters LJ (1994): Epidemic hepatitis E in Pakistan: Patterns of serologic response and evidence that antibody to hepatitis E virus protects against disease. The Journal of Infectious Disease 170:517–521.

Clayson E, Myint K, Snitbhan R, Vaughn D, Innis B, Chan L, Cheung P, Shrestha M (1995): Viremia, fecal shedding, and IgM and IgG responses in patients with hepatitis E. The Journal of Infectious Diseases 172:927–933.

Coursaget P, Krawczynski K, Buisson Y, Nizou C, Molinie C (1993): Hepatitis E and hepatitis C virus infections among French soldiers with non-A, non-B hepatitis. Journal of Medical Virology 39:163–166.

Coursaget P, Depril N, Buisson Y, Molinie C, Roue R (1994): Hepatitis type E in a French population: Detection of anti-HEV by a synthetic peptide-based enzyme-linked immunosorbent assay. Research in Virology 145:51–57.

Cubitt D, Bradley DW, Carter MJ, Chiba S, Estes MK, Saif LJ, Schaffer FL, Smith AW, Studdert MJ, Thiel HJ (1995): Caliciviridae. Archives of Virology 10:359–363.

Dawson GJ, Mushahwar IK, Chau KH, Gitnick GL (1992): Detection of long-lasting antibody to hepatitis E virus in a US traveller to Pakistan [letter]. Lancet 340:426–427.

Dawson GJ, Chau KH, Cabal CM, Yarbough PO, Reyes GR, Mushahwar IK (1992): Solid-phase enzyme-linked immunosorbent assay for hepatitis E virus IgG and IgM antibodies utilizing recombinant antigens and synthetic peptides. Journal of Virological Methods 38:175–186.

Favorov MO, Khudyakov YE, Fields HA, Khudyakova NS, Padhye N, Alter MJ, Mast E, Polish L, Yashina TL, Yarasheva DM, Onischenko GG, Margolis HS (1994): Enzyme immunoassay for the detection of antibody to hepatitis E virus based on synthetic peptides. Journal of Virological Methods 46:237–250.

Gupta H, Joshi YK, Varma A, Shenoy S, Sriramchari S, Iyenger B, Tandon BN (1990): Transmission of enteric non-A, non-B hepatitis virus in Macaca mulatta monkeys by intraportal route: Subsequent passages of HEV virus. Journal of Gastroenterology and Hepatology 5:608–615.

He J, Tam AW, Yarbough PO, Reyes GR, Carl M (1993): Expression and diagnostic utility of hepatitis E virus putative structural proteins expressed in insect cells. Journal of Clinical Microbiology 31:2167–2173.

Heath TCB, Burrow JNC, Currie BJ, Bowden FJ, Fisher DA, Demediuk BH, Locarnini SA, Anderson DA (1995): Locally acquired hepatitis E in the Northern Territory of Australia. The Medical Journal of Australia 162:318–319.

Huang CC, Nguyen D, Fernandez J, Yun KY, Fry KE, Bradley DW, Tam AW, Reyes GR (1992): Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). Virology 191:550–558.

Ichikawa M, Araki M, Rikihisa T, Uchida T, Shikata T, Mizuno K (1991): Cloning and expression of cDNAs from enterically-transmitted non-A, non-B hepatitis virus. Microbiology and Immunology 35:535–543.

Jameel S, Durgapal H, Habibullah CM, Khuroo MS, Panda SK (1992): Enteric non-A, non-B hepatitis: Epidemics, animal transmission, and hepatitis E virus detection by the polymerase chain reaction. Journal of Medical Virology 37:263–270.

Kaur M, Hyams KC, Purdy MA, Krawczynski K, Ching WM, Fry KE, Reyes GR, Bradley DW, Carl M (1992): Human linear B-cell epitopes encoded by the hepatitis E virus include determinants in the RNA-dependent RNA polymerase. Proceedings of the National Academy of Sciences USA 89:3855–3858.

Khudyakov YE, Khudyakova NS, Fields HA, Jue D, Starling C, Favorov MO, Krawczynski K, Polish L, Mast E, Margolis H (1993): Epitope mapping in proteins of hepatitis E virus. Virology 194:89–96.

Khudyakov YE, Favorov MO, Khudyakova NS, Cong ME, Holloway BP, Padhye N, Lambert SB, Jue DL, Fields HA (1994): Artificial mosaic protein containing antigenic epitopes of hepatitis E virus. Journal of Virology 68:7067–7074.

Khudyakov YE, Khudyakova NS, Jue DL, Wells TW, Padhya N, Fields HA (1994): Comparative characterization of antigenic epitopes in the immunodominant region of the protein encoded by open reading frame 3 in Burmese and Mexican strains of hepatitis E virus. Journal of General Virology 75:641–646.

Khudyakov YE, Favorov MO, Jue DL, Hine TK, Fields HA (1994): Immunodominant antigenic regions in a structural protein of the hepatitis E virus. Virology 198:390–393.

Koonin EV, Gorbalenya AE, Purdy MA, Rozanov MN, Reyes GR, Bradley DW (1992): Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: Delineation of an additional group of positive-strand RNA plant and animal viruses. Proceedings of the National Academy of Sciences USA 89:8259–8263.

Krawczynski K (1993): Hepatitis E. Hepatology 17:932–941.

Li F, Zhuang H, Kolivas S, Locarnini S, Anderson D (1994): Persistent and transient antibody responses to hepatitis E virus detected by Western immunoblot using open reading frame 2 and 3 and glutathione S-transferase fusion proteins. Journal of Clinical Microbiology 32:2060–2066.

Lok AS, Kwan WK, Moeckli R, Yarbough PO, Chan RT, Reyes GR, Lai CL, Chung HT, Lai TS (1992): Seroepidemiological survey of hepatitis E in Hong Kong by recombinant-based enzyme immunoassays. Lancet 340:1205–1208.

Longer CF, Denny SL, Caudill JD, Miele TA, Asher LV, Myint KS, Huang CC, Engler WF, LeDuc JW, Binn LN, Ticehurst JR (1993): Experimental hepatitis E: Pathogenesis in cynomolgus macaques (Macaca fascicularis). The Journal of Infectious Diseases 168:602–609.

Mast E, Purcell R, Holland P, Alter M, Group tHEVASPE (1996): Evaluation of assays for antibody to hepatitis E virus by a serum panel. In "IX Triennial International Symposium on Viral Hepatitis and Liver Disease [abstract]." Rome, Italy, p 13.

Panda S, Nanda S, Zafrullah M, Ansari I-H, Ozdener M, Jameel S (1995): An Indian strain of hepatitis E virus (HEV): Cloning, sequence, and expression of structural region and antibody response in sera from individual from an area of high-level HEV endemicity. Journal of Clinical Microbiology 33:2653–2659.

Paul DA, Knigge MF, Ritter A, Gutierrez R, Pilot MT, Chau KH, Dawson GJ (1994): Determination of hepatitis E virus seroprevalence by using recombinant fusion proteins and synthetic peptides. The Journal of Infectious Diseases 169:801–806.

Purdy MA, McCaustland KA, Krawczynski K, Tam A, Beach MJ, Tassopoulos NC, Reyes GR, Bradley DW (1992): Expression of a hepatitis E virus (HEV)-trpE fusion protein containing epitopes recognized by antibodies in sera from human cases and experimentally infected primates. Archives of Virology 123:335–349.

Purdy MA, McCaustland KA, Krawczynski K, Spelbring J, Reyes GR, Bradley DW (1993): Preliminary evidence that a trpE-HEV fusion protein protects cynomolgus macaques against challenge with wild-type hepatitis E virus (HEV). Journal of Medical Virology 41:90–94.

Purdy MA, Tam AW, Huang C-C, Yarbough PO, Reyes GR (1993): Hepatitis E virus: A non-enveloped member of the "alpha-like" RNA virus supergroup. Seminars in Virology 4:319–326.

Reyes GR, Purdy MA, Kim JP, Luk KC, Young LM, Fry KE, Bradley DW (1990): Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. Science 247:1335–1339.

Tam AW, Smith MM, Guerra ME, Huang CC, Bradley DW, Fry KE, Reyes GR (1991): Hepatitis E virus (HEV): Molecular cloning and sequencing of the full-length viral genome. Virology 185:120–131.

Tassopoulos NC, Krawczynski K, Hatzakis A, Katsoulidou A, Delladetsima I, Koutelou MG, Trichopoulos D (1994): Case report: Role of hepatitis E virus in the etiology of community-acquired non-A, non-B hepatitis in Greece. Journal of Medical Virology 42:124–128.

Tsarev SA, Emerson SU, Reyes GR, Tsareva TS, Legters LJ, Malik IA, Iqbal M, Purcell RH (1992): Characterization of a prototype strain of hepatitis E virus. Proceedings of the National Academy of Sciences USA 89:559–563.

Tsarev SA, Emerson SU, Tsareva TS, Yarbough PO, Lewis M, Govindarajan S, Reyes GR, Shapiro M, Purcell RH (1993): Variation in course of hepatitis E in experimentally infected cynomolgus monkeys. The Journal of Infectious Diseases 167:1302–1306.

Tsarev SA, Tsareva TS, Emerson SU, Kapikian AZ, Ticehurst J, London W, Purcell RH (1993): ELISA for antibody to hepatitis E virus (HEV) based on complete open-reading frame-2 protein expressed in insect cells: Identification of HEV infection in primates. The Journal of Infectious Diseases 168:369–378.

Tsarev SA, Tsareva TS, Emerson SU, Govindarajan S, Shapiro M, Gerin JL, Purcell RH (1994a): Successful passive and active immunization of cynomolgus monkeys against hepatitis E. Proceedings of the National Academy of Sciences USA 91:10198–10202.

Tsarev SA, Tsareva TS, Emerson SU, Yarbough PO, Legters LJ, Moskal T, Purcell RH (1994b): Infectivity titration of a prototype strain of hepatitis E virus in cynomolgus monkeys. Journal of Medical Virology 43:135–142.

Tsarev SA, Tsareva T, Emerson S, Rippy M, Zack P, Shapiro M, Purcell R (1995): Experimental hepatitis E in pregnant rhesus monkeys: Failure to transmit hepatitis E virus (HEV) to offspring and evidence of naturally acquired antibodies to HEV. The Journal of Infectious Diseases 172:31–37.

Uchida T, Suzuki K, Hayashi N, Iida F, Hara T, Oo SS, Wang CK, Shikata T, Ichikawa M, Rikihisa T, Mizuno K, Win KM (1992): Hepatitis E virus: cDNA cloning and expression. Microbiology and Immunology 36:67–79.

Yarbough PO, Tam AW, Fry KE, Krawczynski K, McCaustland KA, Bradley DW, Reyes GR (1991): Hepatitis E virus: Identification of type-common epitopes. Journal of Virology 65:5790–5797.

Yarbough PO, Garza E, Moeckli R, Palings I, Velligan M, Trager G, Simonsen C (1993): Assay development of diagnostics tests for hepatitis E. In: "International Symposium on Viral Hepatitis and Liver Disease. [Scientific Program and Abstract].", Tokyo: VHFL, p 87.

Yarbough PO, McCaustland K, Zhang Y, Garcon N, Spelbring J, Carson D, Myriam F, Lifson J, Slaoui M, Prieels JP, Margolis H, Fuerst T (1996): Prevention of hepatitis E using r62K ORF2 subunit vaccine. In "IX Triennial International Symposium on Viral Hepatitis and Liver Disease [abstract]." Rome, Italy p 50.

Zanetti AR, Dawson GJ (1994): Hepatitis type E in Italy: A seroepidemiological survey. Study Group of Hepatitis E. Journal of Medical Virology 42:318–320.